

DIFFERENTIATION OF FIVE SPECIES OF *MEGACHILE* (HYMENOPTERA: MEGACHILIDAE), BASED ON WING SHAPE

Shahram Falamarzi¹
Behzad Habibpour^{1*}
Mohammad S. Mossadegh¹
Alireza Monfared²

¹Department of Plant Protection, College of Agriculture, Shahid Charman University of Ahvaz, Ahvaz, Iran

²Department of Plant Protection, Faculty of Agriculture, Yasouj University, Yasouj, Iran

* corresponding author: habibpour_b@scu.ac.ir
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Abstract

In the present work we used landmark-based geometric morphometrics to compare the wing shapes of five species of *Megachile* (belonging to three subgenera) to confirm whether this technique may be used reliably for differentiation of this group. Analyses of wing shape by the use of principal component analysis (PCA), and canonical variate analysis (CVA) led to a clear differentiation among species. We found a close phenotypic similarity in wing shape between *M. albisecta* (belonging to the subgenus *Creightonella*) and *M. picicornis* (belonging to the subgenus *Eutricharaea*). According to the results of UPGMA, a higher degree of divergence between *M. farinosa* (belonging to the subgenus *Pseudomegachile*) and species belonging to other subgenera, was detected. The results of a cross-validation test indicated that geometric morphometrics is an effective technique to use for distinguishing between *Megachile* species. The reliability rate of this technique was between 85.71-100%. Using only two submarginal cell landmarks for generating shape variables, the cross-validation test correctly assigned individuals to their respective species, with a 92.85-100% reliability rate. Significant differences in wing size were obtained among the analysed species.

Keywords: geometric morphometrics, *Megachile*, species differentiation, wing shape

INTRODUCTION

Megachilidae with more than 4093 described species is the second-largest family of the bees in the world (Michener, 2007; Ascher & Pickering, 2015). These solitary bees include many pollinators of natural, urban, and agricultural vegetation (Bohart, 1972; Michener, 2007; Pitts-Singer & Cane, 2011). *Megachile* is a cosmopolitan genus of this family, containing more than 2000 species and 58 currently recognised subgenera, with a fossil record from Dominican amber (Michener, 2007). Many species of this genus are key pollinators of flowering crops and plants (Sheffield et al., 2011).

The taxonomy of the *Megachile* is a matter of discussion. The number of genera or subgenera recognised in *Megachile* is controversial (Michener, 2007). Gonzalez (2008) examined

the subgenera and suggested a new classification schema using phylogenetic framework for the genus. He explored the relationship of the subgenera of *Megachile* by conducting a cladistic analysis on external morphological characters of the adults. Identification keys of *Megachile* species, particularly females, are constructed based on the *mandibular teeth*. In some specimens, using this character poses difficulties, such as distinguishing the shape and even number of teeth (Sheffield et al., 2011). These problems highlight the need for applying alternative methods to make clear the classification of this group. Clarification of the taxonomic pattern in these bees can be the subject of molecular research. However, in many cases, especially within dried specimens, we cannot use molecular techniques. Recent developments in geometric morphometrics explor-

atory-approaches, generate new perspectives for the assessment of morphological characters in this taxonomic matter (Baylac, Villemant, & Simbolotti, 2003).

Geometric morphometrics are the new way of detecting shape variation and covariation of shape with other factors or variables (Bookstein, 1991). These methods allow a rigorous quantification of morphological structure shapes based on Cartesian coordinates, after removing the effects of all nonshape variation due to position, scale, and rotation (Adams, Slice, & Rolf, 2004). Since the insect wings are rigidly articulated structures they have become an ideal subject for geometric morphometrics studies (Pavlinov, 2001). Wings are often morphologically taxon-specific and show many methodological merits in comparison with other organs (De Meulemeester et al., 2012).

To date, wing geometric morphometrics have been extensively used to resolve the taxonomic dilemma of some species groups. Morphological analyses help with bee identification by defining and extracting homologous landmarks relevant and discriminatory features from the wing images, so that bees can be classified. Geometric morphometrics have been employed successfully to discriminate pollinator bees (Tofilski, 2008; Gerula et al., 2009; Francoy et al., 2009; Francoy, Franco, & Roubik, 2012; Miguel et al., 2011; Oleksa & Tofilski, 2014).

Geometric morphometrics techniques have been used in only a few publications for Megachilids. By using the relative warps, PCA graphs, and UPGMA trees obtained from wing shape differences, Güler, Aytekin, & Cagatay (2006) recognised the classificatory plan in different taxon of Megachilidae. Williams & Goodell (2000) by combining geometric morphometrics methods and phylogenetic comparisons, examined the relationship between the shape of the mandible and material utilised in nest constructing by five *Osmia* Panzer species.

In Iran, geometric morphometric studies have recently been carried out on bees belonging to the genus *Apis* Linnaeus (Kandemir et al., 2009; Dolati, Nazemi Rafie, & Kholesro, 2013; Nazemi Rafie, Mohamadi, & Teimory, 2014).

Since there is no published information on wing geometric morphometrics of *Megachile* species, we conducted a comparative study among five species belonging to three subgenera of *Megachile* including *Pseudomegachile*, *Eutricharaea*, and *Creightonella* (*M. farinosa* belonging to the subgenus *Pseudomegachile*; *M. albisecta* belonging to the subgenus *Creightonella*; *M. anatolica*, *M. minutissima*, and *M. picicornis* belonging to the subgenus *Eutricharaea*). We

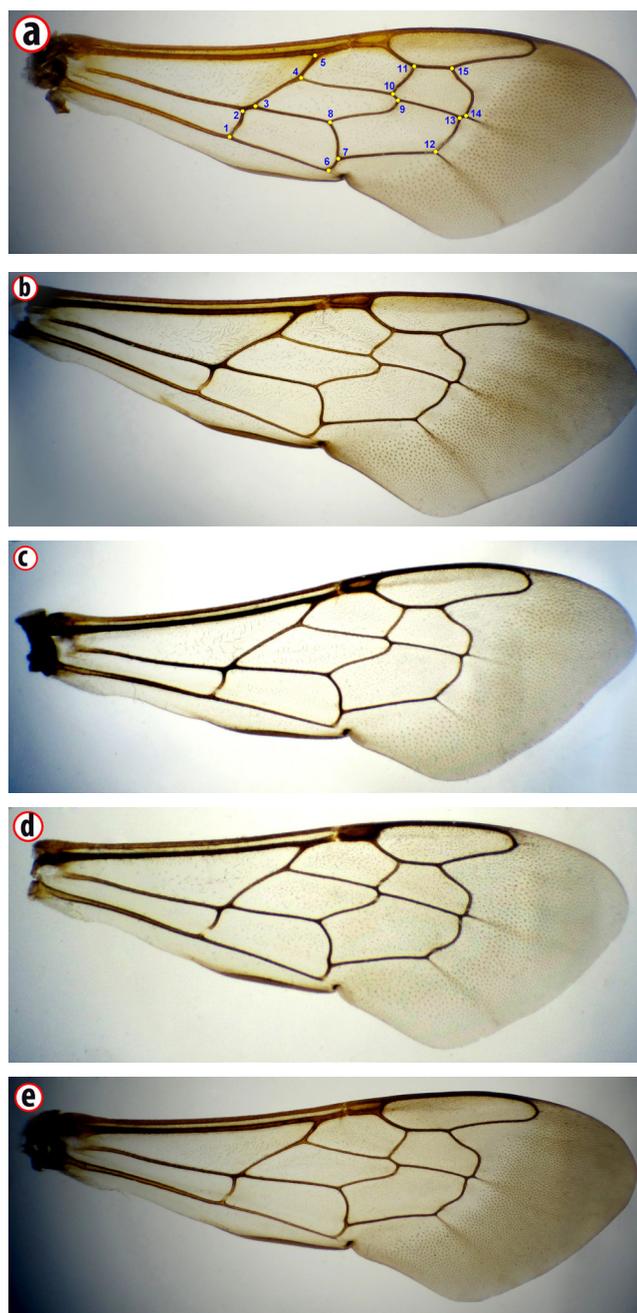


Fig. 1. Right forewings of five species of *Megachile*. (a) Wing of *Megachile farinosa* with the 15 landmarks selected for geometric morphometric analysis (b) Wing of *M. albisecta* (c) Wing of *M. anatolica* (d) Wing of *M. minutissima* (e) wing of *M. picicornis*.

Table 1

Specimen's number of each species included in the analysis

Species	Subgenus	Number
<i>Megachile albisecta</i> Klug, 1817	Creightonella	14
<i>Megachile anatolica</i> Rebmann, 1968	Eutricharaea	17
<i>Megachile farinosa</i> Smith, 1853	Pseudomegachile	21
<i>Megachile minutissima</i> Radoszkowski, 1876	Eutricharaea	20
<i>Megachile picicornis</i> Morawitz, 1853	Eutricharaea	21

examined the forewing shape of these species to determine if this type of information is sufficient to discriminate between the species.

MATERIAL AND METHODS

Ninety-three female specimens of the genus *Megachile* (Tab. 1) were collected from Fars Province, Iran in 2013 and 2014. The right forewings (Fig.1a-e) of each bee were removed and mounted onto a microscope slide and photographed digitally with a camera attached to a stereomicroscope. Fifteen landmarks were plotted at the intersections of the wing veins (Fig. 1a) using TpsDig2.16 software (Rohlf, 2010). For future comparisons, wing photographs of all specimens are available as supplementary online information. The coordinates of landmarks were superimposed using a generalised least square algorithm in MorphoJ 1.06b software (Klingenberg, 2011). All of statistical analyses (except the Tukey test on wing centroid size, creating UPGMA and Forward stepwise discriminant function analysis) were performed in MorphoJ software. We used a One-way analysis of variance (ANOVA) test on wing centroid size, to determine the variation in wing size among different species. The significance of the centroid size pairwise differences among species was tested through the Tukey HSD test and visualised using PAST software (Fig. 2). A multivariate analysis of variance (MANOVA) was used to test the significance of the wing shape differences among species. The shape variations of the wings among species were explicitly based on PCA and CVA. Further statistical computations including Mahalanobis distances, and discriminant function analyses (DFA), were conducted to discriminate between species. The percentages of correct classifications were calculated using

the leave-one-out cross-validation procedure. Forward stepwise discriminant function analysis (tolerance = 0.01; F to enter = 1.0) was used to determine classification functions (Statistica ver. 10.0, StatSoft 2011). The phenetic relationships among species were estimated by UPGMA cluster analysis. Average Mahalanobis distances were used in cluster analysis for construction of a UPGMA dendrogram by PAST program (Hammer, Harper, & Ryan, 2001).

We carried out morphometric analyses on two data sets. The first analysis was based on 15 landmarks defined by the intersections of the wing veins and the second data set resulted from two submarginal cell landmarks (including 4,5,9,10,11,13,14, and 15). From this analysis only, cross-validation procedure results were prepared.

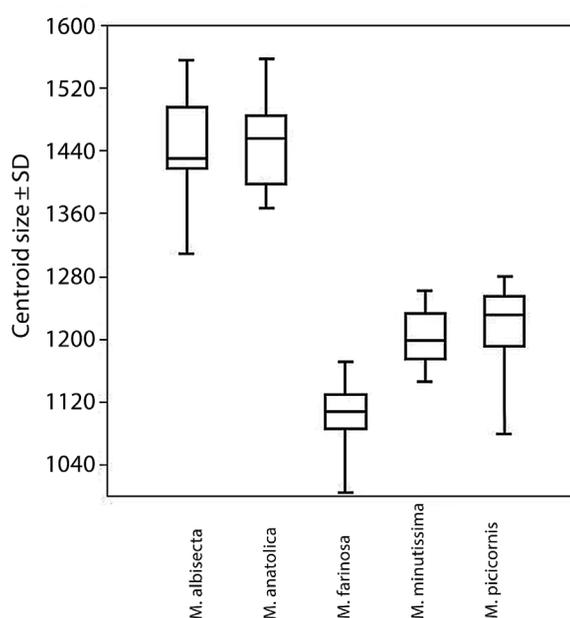


Fig. 2. Centroid size of species of *Megachile* with the mean, standard error and standard deviation illustrating variation in wing size

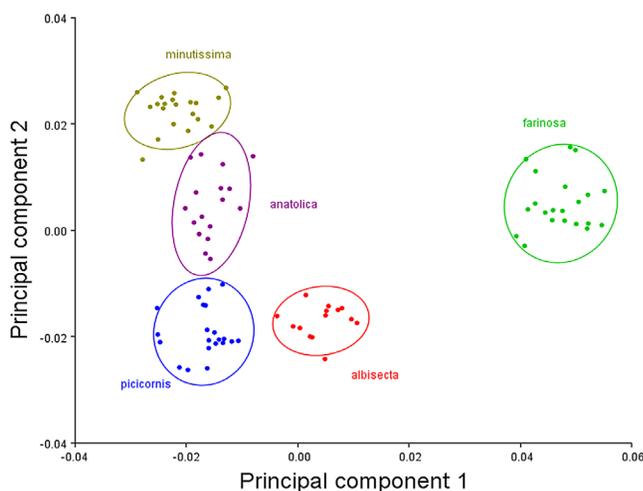


Fig. 3. Distribution of the *Megachile* specimens along the first two principal components based on 15 landmark's data set

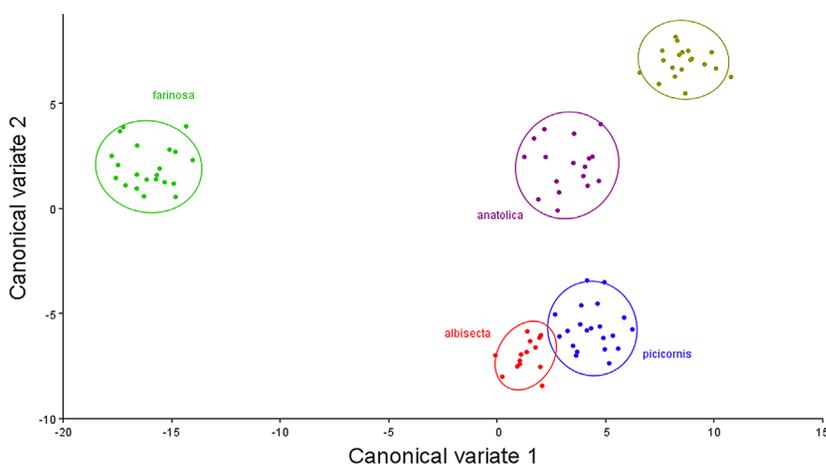


Fig. 4. Distribution of the *Megachile* specimens along the first two canonical variates based on 15 landmark's data set.

RESULTS

Analyses on the data set based on 15 landmarks were defined by the intersections of the wing veins:

A significant size variation ($F_{4,88} = 175.9$, $P < 0.0001$) was found among species. The Tukey HSD test showed a significant difference among all species pair comparisons except *M. minutissima* / *M. picicornis* and *M. anatolica* / *M. albisecta* pairs (Fig. 2).

The principle component analysis of the Cartesian coordinates extracted from the wings of the five species gave nine eigenvalues greater than one, which explained 94.942% of the variation among the species. In the PCA rep-

resentation (Fig. 3), the first two axes explain 50.87 % and 18.46 %, of the variation, respectively. Different species did not show overlap in the scatterplot of the first two principal components. Species belonging to *Eutricharaea* and *Creightonella* subgenera clustered near each other but species of *M. farinosa* belonging to the *Pseudomegachile* subgenus occupy a distinct region.

The canonical variate analysis revealed 4 canonical variates, of which the first three canonical axes accounted for 66.65 %, 22.45 % and 7.50 % of the total shape variation, respectively, and 96.61 % overall. The canonical variate analysis for the wing shape (Fig. 4) shows a good discrimination of each species. Specimens

of *M. farinosa* were separated in a distinct cluster while interestingly, specimens of *M. picicornis* belonging to the subgenus *Eutricharaea* were closer to the individuals of *M. albisecta* belonging to the subgenus *Creightonella* than to other species of the subgenus *Eutricharaea*.

To explore the relationships among the species, pairwise Mahalanobis distances were calculated (Tab. 2). The scores of

the Mahalanobis distances ranged from 8.5782 (*M. albisecta* vs. *M. picicornis*) to 25.2687 (*M. farinosa* vs. *M. minutissima*). Based on the Mahalanobis distances, the largest difference was observed between *M. farinosa* and *M. minutissima*, whereas the lowest Mahalanobis distance was found between *M. albisecta* and *M. picicornis*, as suggested by their close position in the scatterplot of the scores on the first two canonical variates.

According to the discriminant analysis results, all specimens were correctly classified 100 % of the time. The efficacy of classifying individual specimens based on landmark configurations was explored using cross-validation tests on discriminant function analyses of species pairs. The cross-validated reclassification accuracy based on the Mahalanobis distances ranged from 85.71 to 100% among the species (Tab. 3).

Table 2

Mahalanobis Square distances between five species of Megachile

	<i>M. albisecta</i>	<i>M. anatolica</i>	<i>M. farinosa</i>	<i>M. minutissima</i>
<i>M. anatolica</i>	12.9469			
<i>M. farinosa</i>	20.2068	20.2299		
<i>M. minutissima</i>	16.3013	11.0142	25.2687	
<i>M. picicornis</i>	8.5782	9.9617	22.0444	14.2263

Table 3

Results of the discriminant analyses performed on the wing configuration (15 landmarks) according to the leave-one-out cross-validation test. Percent classifications are in parentheses; N denotes the number of species

	<i>M. albisecta</i>	<i>M. anatolica</i>	<i>M. farinosa</i>	<i>M. minutissima</i>	<i>M. picicornis</i>	N
<i>M. albisecta</i>	12 (85.71%)	0	0	0	2	14
<i>M. anatolica</i>	0	15 (88.23%)	0	2	0	17
<i>M. farinosa</i>	0	0	21 (100%)	0	0	21
<i>M. minutissima</i>	0	1	0	19 (95%)	0	20
<i>M. picicornis</i>	1	1	0	0	19 (90.47%)	21

In the cross-validation test, all specimens were correctly assigned to their species, except 7 out of 93 cases which were misclassified in different species: two *M. albisecta* were identified as *M. picicornis*, two *M. anatolica* as *M. minutissima*, one *M. minutissima* as *M. anatolica*, one *M. picicornis* as *M. albisecta*, and one *M. picicornis* as *M. anatolica*.

For creating classification function, coordinates of the 15 landmarks were determined for each specimen and the average configurations were calculated (Tab. 4). The average configuration superimposed on the reference configuration. After superimposition, 23 of 30 variables were selected for the discrimination (Tab. 5). Using classification functions, we can classify an unknown specimen to one of the five species. The superimposed landmarks should be taken together with the discrimination functions to calculate the discriminant score for each species. The coefficients of classification function should be used to multiply the scores of each case on the proper discriminator variables. Then, the products should be added up and the constant value should be added. As a result, the case would have received five discriminant scores.

The specimen should be assigned to the species for which it has the largest calculated discriminant score (Brown & Wicker 2000; Gerula et al. 2009).

The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis of the squared Mahalanobis distances computed from wing shape variables, clustered *M. albisecta* with

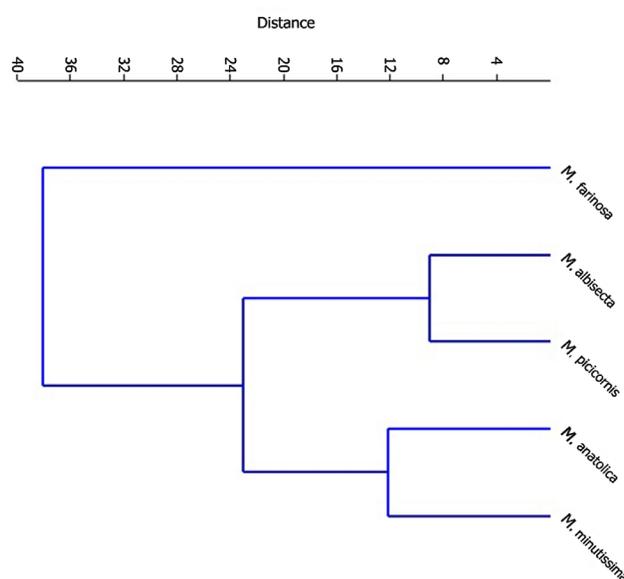


Fig. 5. UPGMA phenogram based on the squared Mahalanobis distances between Megachile species.

Table 4

Mean configuration of the 15 landmarks coordinates for five *Megachile* species

LM	<i>M. albisecta</i>	<i>M. anatolica</i>	<i>M. farinosa</i>	<i>M. minutissima</i>	<i>M. picicornis</i>	All 5 species (reference con- figuration)
1X	-0.39590063	-0.40666403	-0.39452198	-0.40461041	-0.40009224	-0.40061895
1Y	-0.05569811	-0.05452747	-0.05303075	-0.06177397	-0.05768844	-0.05654008
2X	-0.35835951	-0.36420295	-0.34953870	-0.34955671	-0.36217886	-0.35661964
2Y	0.01259464	0.01455039	0.02117334	0.01011591	0.01481911	0.01496665
3X	-0.32614237	-0.33224517	-0.31329671	-0.31672532	-0.33425249	-0.32435764
3Y	0.02599752	0.02785671	0.03275765	0.02440053	0.02462208	0.02734525
4X	-0.17149612	-0.14056669	-0.16484306	-0.16136266	-0.15675655	-0.15886111
4Y	0.10768218	0.12137360	0.10897135	0.11882668	0.11165488	0.11387374
5X	-0.11621119	-0.09895066	-0.11616031	-0.11412268	-0.11506167	-0.11235189
5Y	0.16897691	0.17349851	0.17113894	0.17672403	0.16447293	0.17106523
6X	-0.08093946	-0.07611280	-0.10553140	-0.06812234	-0.07307428	-0.08122530
6Y	-0.17746581	-0.18513592	-0.17354163	-0.19472479	-0.18014968	-0.18254584
7X	-0.04493084	-0.04768773	-0.07416086	-0.04441340	-0.03911791	-0.05072160
7Y	-0.14193224	-0.14838195	-0.14011921	-0.15631928	-0.14615716	-0.14697699
8X	-0.06357293	-0.06797021	-0.07787132	-0.06529016	-0.05925981	-0.06702443
8Y	-0.03677787	-0.03660034	-0.03663090	-0.03373603	-0.03480942	-0.03569914
9X	0.11914697	0.11734579	0.12862004	0.10719295	0.11613185	0.11780427
9Y	0.02276003	0.02660528	0.02034885	0.03377026	0.02189687	0.02516711
10X	0.09269409	0.09962248	0.10994605	0.09186446	0.09262834	0.09775644
10Y	0.04918700	0.04859426	0.04367070	0.05556885	0.04492188	0.04834373
11X	0.16347073	0.16762396	0.18216267	0.15793457	0.15600183	0.16570935
11Y	0.11804178	0.12318956	0.11846377	0.12779506	0.12221400	0.12227444
12X	0.22240059	0.21974383	0.23001110	0.22911361	0.22005758	0.22464104
12Y	-0.13414903	-0.14276889	-0.14093735	-0.14426520	-0.13141223	-0.13891427
13X	0.32208914	0.31376235	0.31511962	0.32394201	0.32689200	0.32065332
13Y	-0.03497812	-0.04119457	-0.04252044	-0.03884812	-0.03525656	-0.03887373
14X	0.34555054	0.33523505	0.33077524	0.34418405	0.34798691	0.34078133
14Y	-0.03069968	-0.03437167	-0.03754848	-0.03142229	-0.02914741	-0.03291152
15X	0.29220099	0.28106678	0.29928963	0.26997202	0.28009531	0.28443483
15Y	0.10646080	0.10731250	0.10780415	0.11388835	0.11001915	0.10942544

M. picicornis and *M. anatolica* with *M. minutissima* in the same branch, while *M. farinosa* was the most divergent species in a very distinctive clade (Fig. 5).

Analyses based on 8 landmarks of two submarginal cells:

The landmarks 13 and 14 related to the second submarginal cell are determined as having the two highest relative contributions to the variation in shape of the forewing (with values of SS=0.28433 and 0.22756, respectively). For this reason, we performed another analysis on the data set resulted from two submarginal cell landmarks. The cross-validated reclassification accuracy based on the Mahalanobis

distances, ranged from 92.85 to 100% among the species (Tab. 6). In the cross-validation test, all specimens were correctly assigned to their species, except 3 out of 93 cases which were misclassified as different species: one *M. albisecta* was identified as *M. picicornis*, one *M. minutissima* as *M. anatolica*, and one *M. picicornis* as *M. anatolica*.

DISCUSSION

In the present study, a landmark-based geometric morphometrics technique was applied to study the wing shape variables among five species of *Megachile* belonging to three subgenera. This is the first study which investigated the

Table 5

Classification functions for discrimination of five *Megachile* species

Variable	<i>M. albisecta</i>	<i>M. anatolica</i>	<i>M. farinosa</i>	<i>M. minutissima</i>	<i>M. picicornis</i>
7X	-441689	-445685	-453778	-445444	-442449
12Y	-519675	-519197	-519572	-520835	-517903
2X	-853382	-851816	-859156	-849114	-851411
6X	-642077	-636927	-641505	-636714	-639310
2Y	-63125	-59012	-53579	-61993	-60406
14X	201261	199067	194886	202572	200262
7Y	-385723	-384959	-387482	-387975	-386068
5X	-325613	-324773	-330445	-323721	-324310
4X	-759829	-756801	-761410	-756633	-757048
10X	-232560	-230713	-232134	-229380	-231454
13Y	-627588	-629784	-634570	-629863	-628680
8X	-441664	-439438	-444281	-437949	-439302
13X	49830	47670	45002	48879	50265
9Y	137157	140188	141063	140245	137755
12X	-25792	-26484	-27281	-25178	-26974
15X	102619	100677	100510	101506	101834
10Y	-215538	-215247	-220479	-213654	-215113
5Y	57510	57273	56446	58803	56605
8Y	-201140	-200786	-203160	-199077	-199385
3Y	330901	329096	327811	333076	331344
1X	-876509	-874317	-877851	-875799	-874367
3X	-832005	-829238	-832280	-829884	-829767
6Y	-408332	-406293	-407166	-406093	-406162
Constant	-759912	-754236	-759713	-757066	-755430

Table 6

Results of the discriminant analyses performed on the wing configuration (8 landmarks) according to the leave-one-out cross-validation test. Percent classifications are in parentheses; N denotes the number of species

	<i>M. albisecta</i>	<i>M. anatolica</i>	<i>M. farinosa</i>	<i>M. minutissima</i>	<i>M. picicornis</i>	N
<i>M. albisecta</i>	13 (92.85%)	0	0	0	1	14
<i>M. anatolica</i>	0	17 (100%)	0	0	0	17
<i>M. farinosa</i>	0	0	21 (100%)	0	0	21
<i>M. minutissima</i>	0	1	0	19 (95%)	0	20
<i>M. picicornis</i>	0	1	0	0	20 (95.23%)	21

systematic relationships within this genus by a statistical quantification of the wing shape differences.

A principle component analysis of wing shape based on dataset of 15 landmarks was appropriate for discriminating between different species. A canonical variate analysis of wing shapes showed that species can be separated clearly from each other. However, we found a very close phenotypic similarity of wing geometry between *M. albisecta* belonging to the subgenus *Creightonella* and *M. picicornis* belonging to the

subgenus *Eutricharaea*. These two species had the most similar wing shape morphology. It was seen by PCA, CVA, and UPGMA graphs that there was a higher phenetic distance of the *M. farinosa* belonging to subgenus *Pseudomegachile* from the other species. In a recent study by Gonzalez (2008), *Pseudomegachile* was considered as a subgenus of the distinct genus *Chalicodoma*. According to his study, *Chalicodoma* is monophyletic as is the sister group of *Megachile*. For elucidating the role of wing geometry in taxonomy of *Megachile*, we can accentuate the

shape of wing cells, particularly the submarginal cell, as morphological markers for discriminating between species. In the *Pseudomegachile* subgenus, the first submarginal cell is longer than the second ones. Because of the obvious difference in size of the two submarginal cells of *M. farinosa*, we propose that the most informative cells for discrimination of this species are submarginal cells. By using only two submarginal cell landmarks for generating shape variables, the cross-validation test correctly assigned individuals to their respective species, with a reliability rate between 92.85-100%.

Current identification keys of *Megachile* females are based on mandibular dentition. Sometimes in old specimens, number and *shape of mandibular teeth* are difficult to distinguish. We had a similar problem identifying specimens with the mandibles closed or dirty specimens (Sheffield et al., 2011). Therefore, we tried to use an alternative classification method in which dentition is not largely relied on. It seems that morphological difference in forewing shape could be indicative of the correct systematic relationships among the species of *Megachile*.

Assessing the decline of the pollinator bees is one of the vital priorities considered by pollination experts. Such an assessment is needed to provide a detailed analysis of the pollinator diversity status and to provide information suitable for enhancing their conservation. Reliable assessment depends on the exact taxonomic identification of these insects. Bees taxonomic capacity is currently inadequate and improvement is critical for their conservation (Eardley et al., 2006). Because there are few taxonomic experts and because of conventional taxonomy, the identification of *Megachile* using geometric morphometrics can help the recognition of these important pollinators to be made more quickly.

This study validates the use of wing traits for *Megachile* species identification. Geometric morphometrics provides a powerful tool to face the insufficiency of taxonomic experts and data that will help in the correct identification of these important pollinators based only on wing geometry. Exact recognition will promote

studies on their ecology and will help focus conservation efforts on these pollinators.

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